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RESEARCH ARTICLE

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Development and characterization of gelatin-based scaffolds for 3D cancer cell culture

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Abstract

Two-dimensional (2D) cell culture is a commonly utilized method in laboratories for growing and maintaining cells, particularly in cancer research under controlled conditions. A critical factor in cancer cell culture is preserving cell viability and functionality, as cancer cells are highly sensitive to changes in their environment. However, the three-dimensional (3D) structure of tumors cannot be effectively replicated in 2D cell culture. In contrast, 3D cell culture provides significant advantages over traditional 2D systems, primarily by cultivating cancer cells in an environment that more accurately resembles the 3D architecture and complexity of tumors in vivo. This study is designed to develop cancer-specific gelatin-based scaffolds for developing a 3D (spheroid) culture model. These scaffolds were prepared reproducibly using pig skin gelatin, cold-water fish gelatin, and bovine skin gelatin. Phytagel and agarose-based gels (control groups) were used for comparison. The scaffold structures were characterized by Fourier transform infrared (FT-IR) spectroscopy and scanning electron microscopy (SEM). MCF-7, HeLa, and HT-29 cancer cells were seeded on gelatin substrates and imaged by inverted microscopy. FT-IR analysis showed that the scaffolds were successfully prepared, while SEM analysis showed that the scaffolds were highly porous. The cancer cell lines were successfully grown on scaffolds and were shown to aggregate to form spherical structures. In this study, for the first time, 3D structures were generated from monolayer cell structures by using different gelatin structures. Many 3D cell studies will benefit from the fact that the resulting gelatin scaffolds are biocompatible and support infiltration and proliferation.

Keywords: Spheroid; Gelatin; Scaffold; Biomaterial; 3D culture; Cancer

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1. Introduction

Cancer refers to a group of diseases marked by the uncontrolled growth and spread of abnormal cells within the body. Advancing cancer research is essential for developing new and effective treatments, enhancing patient outcomes, and ultimately finding a cure for this disease [1].

For a wide range of basic and preclinical studies, cell culture systems are essential [2]. This system is popular among cancer researchers because it allows them to cultivate cells on a plane and examine the essential media for cell maintenance and development under a well-controlled setting [1]. Traditional cell culture methods utilize 2D monolayers that enable in vitro investigation of cellular processes [2–3]. However, with further investigation of the cellular environment, it has become clear that cellular functions in 2D culture systems are significantly different from those observed in the living organism [4]. In this way, it can be said that cells cultivated on 2D surfaces, as compared to the cells that grow in an in vivo environment, are different in terms of morphology, signaling, proliferation, cell-matrix and cell-cell interactions, differentiation and many other parameters [5]. For this reason, it appears that the next frontier in cell biology research is the development and employment of three-dimensional (3D) culture technology that aims to further replicate the in vivo cell microenvironment [6]. A wide range of detailed studies have been conducted in recent years in the direction of advanced 3D culture techniques to achieve a nurturing environment. Their biodegradability further makes them advantageous for tissue engineering applications [7]. Conventional 2D cell cultures cannot adapt to the cellular diversity of both normal and cancerous tissues, making them inadequate for studying tissue-specific processes or diseases such as cancer [2]. 3D cell cultures provide more refined models by simulating cell-cell and extracellular matrix (ECM) interactions and enhancing cell signaling and proliferation [8]. 3D cell culture allows cells to grow in an environment that closely mimics their natural environment, allowing them to interact within a supporting matrix [9].

Scaffolds are essential in 3D cell culture systems as they provide a 3D environment for cells to grow and interact with each other and their environment. Many biomaterials including natural polymers (such as collagen, fibrin), synthetic polymers, decellularized tissues, hydrogels, and microfluids are used to create scaffold-based 3D culture models [1]. Properties of biopolymers such as wall morphology, pore structure and surface area enable the production of scaffolds that support cell seeding, growth and tissue development. Natural polymers function as bioadhesive and biofunctional materials by offering advantages such as biocompatibility, biodegradability, low toxicity and increased cellular interaction [10]. These materials have some advantages and disadvantages. While natural polymers promote cell attachment, proliferation and differentiation with their ECM-like structure, they may have limitations in terms of mechanical strength and stability. Synthetic polymers may require surface modifications for biocompatibility while providing precise control over porosity, degradation rate and mechanical strength. Decellularized tissues retain tissue-specific signals and mechanical properties, but their preparation can be complex and costly [1]. Organoids, another model used to create 3D structures, are clusters of cells derived from stem cells or organ-specific progenitor cells that exhibit organ-like properties. However, these systems have some limitations: lack of vascular structure prevents oxygen and nutrients from reaching the center of the organoids, lack of blood vessels limits the size of the organoids and prevents them from meeting the needs of the tissues, immune and stromal cell interactions in the tumor microenvironment cannot be adequately mimicked [8]. Encapsulating cells within hydrogels is a widely used method for 3D culture. These hydrogels are composed of loosely cross-linked natural materials, such as collagen, agarose, fibrin, or hyaluronic acid, and feature a high water content [11]. Often regarded as the 3D equivalent of native ECM, hydrogels exhibit swelling properties, low elasticity, and high water content. Hydrogels are hydrophilic, three-dimensional cross-linked polymer networks featuring interconnected microscopic pores, allowing them to absorb up to 99% of their volume in biological fluids. As synthetic extracellular matrix scaffolds, they offer structural support and promote spatial organization, enhancing cellular interactions with their surrounding environment [12]. Due to their porous structure, hydrogels support nutrient and oxygen diffusion and ensure cell survival. Their mechanical properties, degradation rate and transport capacity for bioactive molecules can be easily tuned. However, their lack of long-term stability requires careful design and optimization [1].

Gelatin is a gelling agent derived from animal collagen, characterized by its tasteless, translucent, and pale yellow or colorless appearance. It is a high molecular weight biopolymer produced through protein hydrolysis. Its unique amino acid composition, rich in Gly-Pro-Hyp (glycine, L-proline and 3-hydroxy-L-prolin), grants gelatin distinctive functional properties, including gelling, binding, viscosity enhancement, and film-forming abilities [13]. Since the emergence of biomaterials for tissue regeneration, gelatin has been incorporated into various systems, such as injectable hydrogels, scaffolds, and drug delivery platforms [14]. Its advantageous features–such as low toxicity, biocompatibility, and biodegradability–support enhanced differentiation, cell proliferation, and adhesion by body enzymes (e.g., metalloproteinases) without eliciting an immune response. Additionally, its cost–effectiveness has facilitated its use in diverse applications, including hydrogels for sustained chemotherapeutic drug delivery and for promoting bone regeneration across tissues like bone, neural, and skeletal systems. Gelatin's properties, such as water absorption, porosity, support for healthy cell growth, and oxygen transport, are critical for successful tissue regeneration [15].

Phytagel is a polysaccharide with various functions in the production of hydrogels and scaffolds [16]. Phytagel is derived from a bacterial substrate composed of rhamnose, glucuronic acid, and glucose. This polymer consists of repeating tetrasaccharide units that gelate in the presence of mono- or divalent cations [17].

Agarose is a biocompatible and biodegradable biomaterial due to its neutral polysaccharide structure based on D-galactose. These properties make it advantageous for use in tissue engineering and drug delivery. It can be mixed with other biomaterials to create biological scaffolds that can adapt to different soft tissues and can be used in clinical applications such as human skin and organ production [10]. Since its size and stiffness are adjustable, it can mimic natural tissue stiffness in tumor engineering. It also supports cell proliferation, maintains phenotype, and provides a stable cellular environment by increasing ECM secretion [18].

Gelatin can be crosslinked by chemical or enzymatic methods; however, these methods require non-physiological conditions and are not suitable for 3D cell encapsulation. Therefore, practical methods to obtain gelatin-based hydrogels are being investigated, especially to regulate the pore size, degradation, swelling, and mechanical properties of the scaffold [19]. Gelatins from different sources contain various protein and biopolymer components that can optimize cell adhesion and growth, allowing the production of hydrogels with various physical, chemical and biological properties [19–20]. Biomaterials such as cold-water fish gelatin are immunogenic, non-toxic and biodegradable [20]. Hydrogels derived from bovine skin gelatin have favorable mechanical properties that support cell growth, migration and proliferation and offer tunability for the culture of different cell types. Gel stiffness differs between bovine and porcine skin gelatin. While the gel strength of BSG increases proportionally with pH, this increase is inconsistent for pigskin gelatin. Lower stiffness bovine skin gelatin hydrogels are expected to promote cell spreading [19].

The scaffold materials selected for this study are known to exhibit high porosity, thus increasing cell infiltration and enhancing intercellular interactions [20]. In this study, for the first time, 3D structures were formed from monolayered cell structures by using three different gelatin structures obtained from different organisms that contain gelling properties in their structure. These gelatin structures were layered on 96- well plates at specific concentrations, and the most suitable ground for 3D cell structures was prepared and characterized. We fabricated gelatin-based scaffolds with highly homogeneous pore size and biocompatibility using different base materials in this study. Compared to other gel systems previously used for tumor models, the materials used in present study combine the advantages of both natural and synthetic biomaterials, aiming to create more realistic and reliable 3D environments for cell culture studies.

2. Materials and methods

2.1. Materials

Sigma-Aldrich, Merck (Darmstadt, Germany) and/or Fluka (Milwaukee, USA) were purchased for the chemical solvents and substances used in the study. Purified water from a water purifier (Millipore Milli-Q Plus) was used for all aqueous solutions. The pH measurements were carried out using a digital pH meter (Orion 2 Star pH Benchtop). Spheroid cells were photographed with a digital camera (Leica 475) under an inverted microscope (Leica DMI 4000B, Germany).

2.2. Preparation of TAE (Tris-Acetic Acid-EDTA) buffer

First, 48.4 g of Tris was dissolved in 800 mL of distilled water. 11.4 mL of acetic acid was added to the mixture under a fume hood. Add 3.72 g of EDTA and mix well. Finally, 1 L of distilled water was added [21].

2.3. Preparation of basic gelatin for spheroid cell formation

To produce spheroids, we added 80 µL of different gelatin, phytagel and agarose solutions to the bottom of a 96-well plate. We first dissolved 1% phytagel [22], 1% agarose [22], and 1.5% bovine skin, 5% cold water fish, and 10% pig skin gelatin solutions in TAE buffer prepared specifically for this procedure. After preparation, we sterilized the solutions under UV light. To avoid air bubbles, we carefully dispensed the sterilized gel solutions into the wells and evenly spread them across the bottom surfaces. Finally, we placed the plates at 4°C for 10 minutes to allow gel formation [23–24].

2.4. Fourier transform infrared (FT-IR) spectroscopy

The structure of five different gelatin scaffolds prepared for 3D cell formation was analyzed using FT-IR (PerkinElmer FT-IR Spectrum One, USA). The scaffolds were placed in the FT-IR device for analysis, and scanning was conducted across a range of 400 cm^{-1} to 4000 cm^{-1} to detect changes in the functional groups within the 3D gelatin scaffolds [21].

2.5. Scanning electron microscopy (SEM)

We used SEM to understand the morphology and surface properties of agarose, phytagel, and other gelatin structures. For the SEM images, we first coated the materials with a 5 nm layer of Au and then imaged them at a distance of about 1 micron. We used the images to evaluate morphology, porosity, structure, and other important features of the sample [25]. We then analyzed the obtained images with ImageJ image processing software and measured the average porosity and pore size [26]. Using the software, we calculated the pore size by measuring the pore diameters of each scaffold type and the percentage porosity by measuring the area occupied by the pores.

2.6. Preparation of monolayer cell lines

Cell culture studies were performed with MCF-7 (breast cancer line) (ATCC® HTB-22™), HeLa (uterine cancer line) (ACC 733, DSMZ) and HT-29 (colon cancer line) (ATCC® CRL1573™). Cells stored frozen at -80 °C and/or in liquid nitrogen in cold stabilized vials were thawed rapidly in a water bath (at 37 °C). They were placed in the medium after centrifugation for 5 minutes at 1300 rpm. The cancer cells were then incubated in flasks with 1%

penicillin-streptomycin, 10% FBS, 0.01% gentamicin and DMEM with 10% FBS, 1% penicillin-streptomycin and RPMI with 0.01% gentamicin in humidity-controlled incubators at 37 °C with a 5% CO_2 atmosphere. Cells that reached 80-90% confluence in culture were passaged and reseeded [25–27].

2.7. Spheroid cell formation from monolayer cell lines

In spheroid cell studies, passaged cell pellets were seeded in fresh medium at same concentrations (5.0×10^4 cells per mL). This seeding density has been reported as optimal for initiating spheroid formation, allowing sufficient cell–cell contact to promote aggregation [28]. Furthermore, maintaining this moderate cell density ensures that the scaffold porosity and surface area are not overwhelmed, allowing for uniform spheroid morphology and facilitating comparative analyses among different scaffold types. These cells were seeded in well plates containing phytagel, agarose, and different gelatins. In general, in 3D cell culture studies, at least 3 to 7 days are required for cells to attach to the scaffold, proliferate, and form spherical structures [29]. Therefore, cells were cultured for a period of seven days and samples were then analyzed on day 7. Images of the spheroidal cells were captured and recorded using a digital camera (Leica 475) under an inverted microscope (Leica DMI4000B, Germany) [27].

2.8. Statistical analysis

Multiple samples (n=3–6) were collected in each experiment and expressed as mean ± SD. Each experiment was replicated three times. One-way analysis of variance (ANOVA) was used to evaluate statistical significance. *P* values ≤ 0.05 were considered significant.

3. Results and discussion

In this study, we used only physical gelation method for 3D encapsulation of cells and did not resort to chemical cross-linking agents. Agarose, gelatin, and phytagel scaffolds of homogeneous size and shape were successfully and reproducibly produced (Figure 1). It has been observed that the structures obtained by physical gelation have high biocompatibility and provide sufficient support for cell proliferation and global organization.

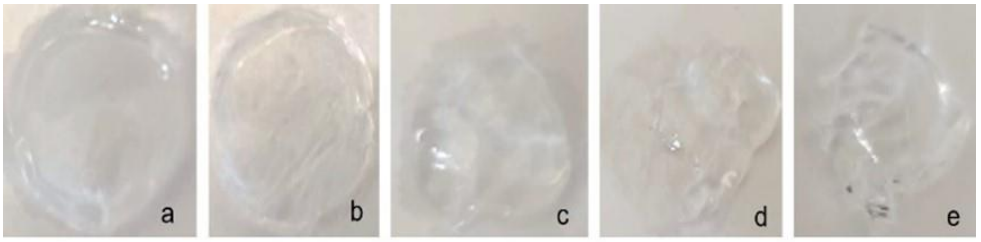

Fig. 1. Macroscopic images of the scaffold structures composed of different sources. (a) Agarose; (b) Phytagel; (c) Bovine skin gelatin; (d) Cold water fish gelatin; (e) Pig skin gelatin.

FT-IR spectroscopy clearly reported the structure of each gelatin composition, and the FT-IR spectrum of each structure obtained is shown in Figure 2. In the FT-IR spectra of gelatin, the C=O stretching at 1630–1635 cm^{-1} was for amide I and the N–H deformation at 1500–1540 cm^{-1} was for amide II. The absorption of amide I was mainly due to the stretching vibration of C–O bond, and the band of amide II was due to the combination of bending of -NH bond and stretching of C–N bond. The band due to N–H stretching was scattered at 3250–3300 cm^{-1} [30–31]. The amide structures obtained by FT-IR analysis indicate that the gelation process has been successful. The agarose hydrogel

(black line in Fig. 2) showed typical signals at 1043 cm^{-1}, corresponding to the C–H bending and C–O stretching of glycosidic bonds, and two broad peaks at 1625 and 3350 cm^{-1}, characteristic of the H–O–H bonded water and O–H hydrogen-bonded hydroxylic groups of carbohydrates, respectively [32]. The FT-IR spectrum of phytagel (red line in Fig. 2) shows a broad absorption band registered at 3320 cm^{-1} specific to the stretching vibrations of the OH groups of the glucopyranose ring. The absorption bands at 1603 cm^{-1} is assigned to asymmetric and symmetric stretching of the COO^- group. Also the vibrations of C–O and C–O–C bonds are present in the region of 1300–1020 cm^{-1} [33].

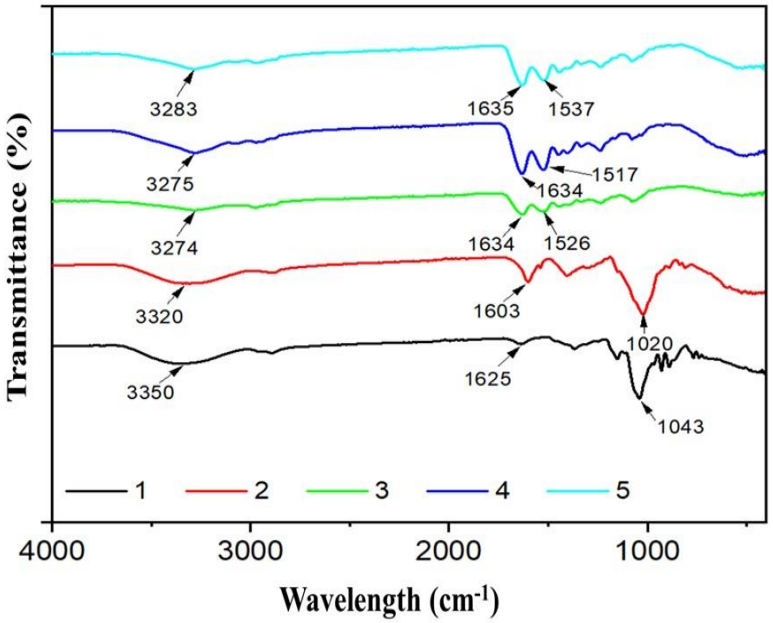

Fig. 2. Fourier-transform infrared (FT-IR) spectra of the fabricated 3D scaffold structures. FT-IR analysis was performed to evaluate the chemical composition and functional groups present in the scaffolds prepared from five different biopolymer sources: (1) Agarose; (2) Phytagel; (3) Bovine skin gelatin; (4) Cold water fish gelatin; (5) Pig skin gelatin. Characteristic absorption peaks corresponding to hydroxyl (–OH), amide (–CO–NH), and carboxyl (–COOH) groups were observed, confirming the presence of expected functional groups inherent to polysaccharide- and protein-based materials. The results validate the successful incorporation of each biopolymer into the scaffold matrix.

Different pore sizes and interconnecting shapes affect the physicochemical properties of the scaffold [34]. Previous studies have shown that the greater the variation in pore size of 3D tissue engineering scaffolds, the more difficult it is for cells to seed and proliferate [35]. SEM was used to observe the morphological changes of the 3D scaffolds. SEM images showed that the scaffolds had an isotropic pore structure and were highly porous (Figure 3). Homogenous microstructure and high porosity of the scaffolds were observed for all scaffold groups. There is a good connection between the pores and the pores have an optimum distribution all over the scaffold surfaces. The inherently interconnected structure of the pores, without a closed-cell configuration, ensured connectivity between adjacent pores.

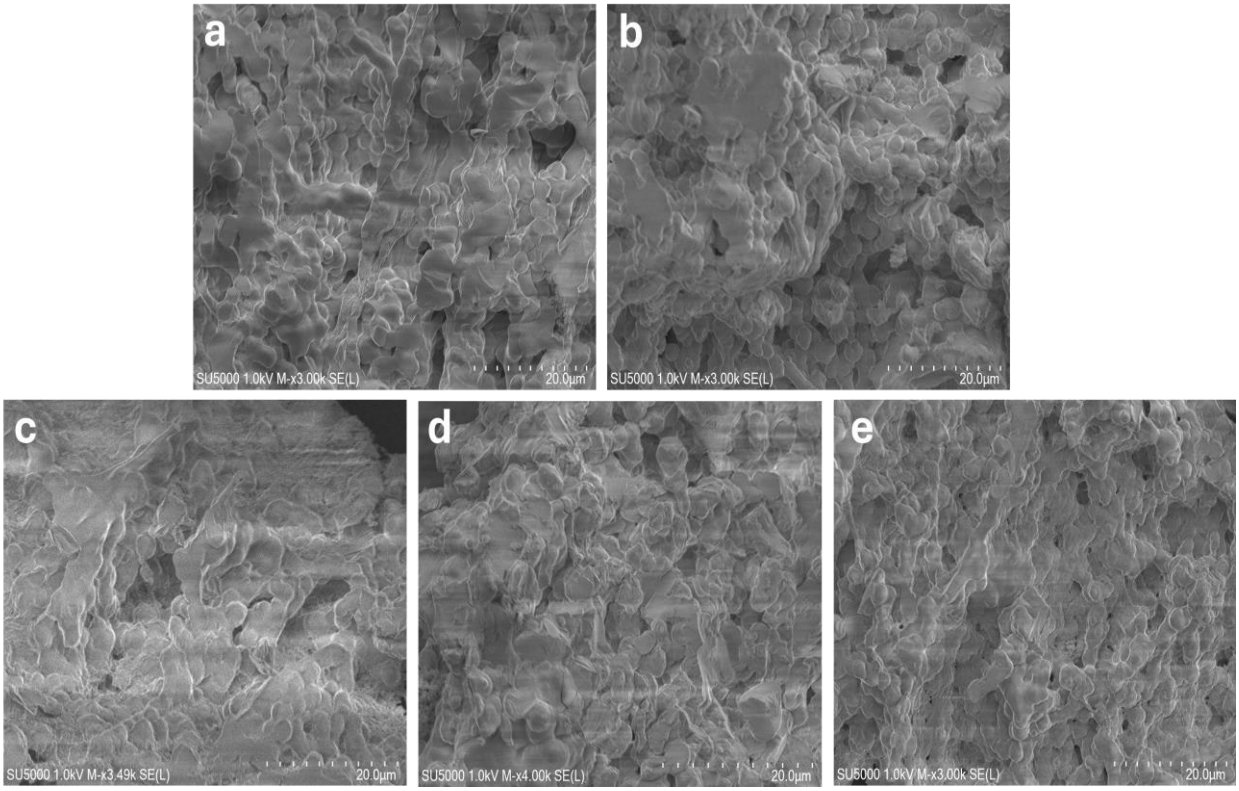

Fig. 3. Scanning electron microscopy (SEM) images of the surface morphology of the prepared 3D scaffolds at ×3000 magnification. All scaffold structures exhibited a highly porous and interconnected morphology with an isotropic pore distribution. The micrographs reveal homogenous pore organization across the surfaces of the scaffolds. The pore interconnectivity and uniform size distribution observed in all groups indicate successful scaffold fabrication under controlled conditions. (a) Agarose; (b) Phytagel; (c) Bovine skin gelatin; (d) Cold water fish gelatin; (e) Pig skin gelatin.

Pores larger than 100 μm enable tissue growth, while extremely large pores (> 400 μm) reduce the cell-cell contact rate because cells exhibit a 2D growth pattern instead of a 3D organization on the substrate [36]. The pore sizes of agarose, phytagel, bovine skin gelatin, cold water fish gelatin and pig skin gelatin were 262.6±39, 237.0±22, 313.0±26, 273.0±39, and 264.4±37 μm, respectively (Figure 4), and these pore sizes are suitable for the formation of 3D cells. The data obtained showed that there was no statistically significant difference between the different scaffolds in terms of mean pore size ($P > 0.05$). Pore sizes were uniform across both transverse and longitudinal planes, with no significant differences observed. However, pore sizes along the longitudinal axis were significantly larger ($P \leq 0.05$) than those in the transverse plane, resulting in oval-shaped pores. This structure may allow cells to be more easily oriented (cell alignment) and may have a potential impact on tissue organization. These findings are consistent with previous findings [37]. Porosity analysis (Figure 4) revealed that all compositions exhibited high porosity levels (> 90%) across all groups (agarose 91.62±1.05, phytagel 90.58±1.25, bovin skin gelatin 91.4±2.13, cold water fish gelatin 91.42±1.381, and pig skin gelatin 91.06±0.823%) with no statistical significance ($P > 0.05$). The findings show that 3D scaffolds with an isotropic pore structure and high porosity create a favorable microenvironment for intercellular connections. The strong intercellular connectivity and the absence of a closed cell structure allow cells to move freely within the scaffold, resulting in highly compliant scaffold [38]. Compared to other studies, these findings are

consistent with some important design principles widely recognized in the literature. For example, Liu et al. showed that scaffolds with high porosity allow for better cellular proliferation and differentiation [39]. Similarly, Jones et al. highlight that the pore structure, pore diameter and connectivity of scaffolds are critical for cells to successfully settle on the scaffold surface and penetrate deep into the scaffold [40]. Additionally, porous scaffolds formed with the combination of fish gelatin and hyaluronic acid had porosity values ranging from 71.72% to 91.17% and supported the proliferation and infiltration of human fibroblast and keratinocyte cells [41]. In conclusion, these findings reveal that scaffolds with high porosity and homogeneous pore structure can potentially outperform in tissue engineering applications and support the successful integration of cells into the scaffold. Consistent with other studies, these results provide important clues for improving cellular performance and designing more functional 3D scaffolds for biomedical applications.

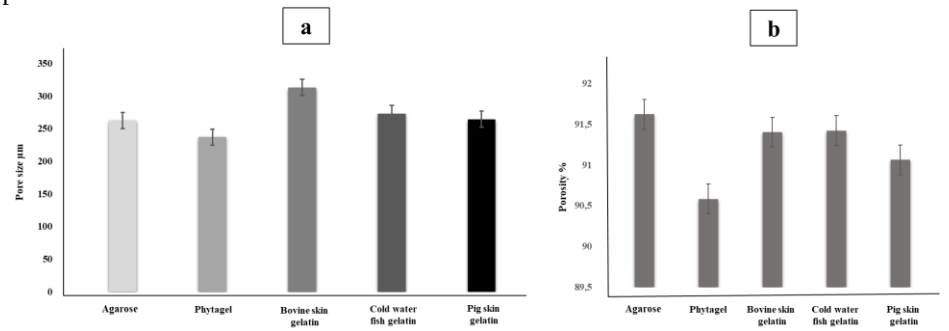

Fig. 4. Quantitative comparison of (a) average pore sizes, and (b) porosity % among different scaffold types used for 3D cell culture applications. Statistical analysis revealed no significant difference in average pore size, and porosity % between the scaffold types ($P > 0.05$), indicating that all scaffold groups exhibit comparable high porosity in terms of dimensional characteristics.

3D cell culture is achieved by embedding cells within artificial matrices, which can consist of solid scaffolds (like foams or sponges), hydrogels, fibers, or beads. These matrices are designed with adjustable porosities and mechanical characteristics to mimic the ECM of tumors in vivo [42]. After seeding, cells adhere to the matrix material and migrate inward, creating microstructures within its cavities.

Artificial matrices for 3D spheroid formation are typically composed of non-adhesive polymers, such as agarose or hyaluronic acid, or a combination of non-adhesive polymers with other biomaterials. Non-adhesive properties are essential for promoting spheroid formation, as they allow cell-cell adhesive forces to dominate over cell-matrix interactions, thereby driving cell aggregation [43]. Researchers have developed and utilized numerous scaffold-based materials for 3D cell culture. In this study, for the first time, we used pig skin gelatin, cold-water fish gelatin, and bovine skin gelatin as base materials for spheroid cell formation. Researchers have employed various base materials such as agar, agarose, and polymer structures for 3D cell formation, and they consider these materials among the most promising for promoting spheroid cell growth. Nevertheless, materials that rely on spheroid formation on flat, non-adhesive surfaces face challenges, such as limited reproducibility. As a result, researchers are exploring different gelatin-based materials to enhance spheroid production and optimize their suitability as base materials.

For comparison, three different cancer cell lines were seeded on these scaffolds. After 7 days, all cell lines formed multicellular spheres. Figure 5 illustrates the 3D structures formed by MCF-7 (4A), HT-29 (4B), and HeLa (4C) cell lines. The reasons for choosing these cancer cell lines are that they each represent common cancer types of different tissue origins and that the behavior of these cells in 2D and 3D cultures is well described in the literature. Thus, the general applicability of the 3D culture model for different cancer types could be evaluated. Morphological analysis using an inverted microscope revealed that the cells predominantly displayed a 3D round morphology (multicellular spheroids), with evident 3D cell-cell and cell-matrix interactions. When we compared gelatin-based scaffolds, we found that bovine skin gelatin-based scaffolds showed the best performance. The possible reason for this may be that

bovine skin gelatin supports cell spreading due to its lower hardness [19]. All grown tumor spheroids are of similar shape with different sizes. The difference could be attributed to the preferences of different cell lines in the tumor microenvironment [44]. Furthermore, the most optimal 3D scaffold structures occurred in the MCF-7 cell line (bovine skin gelatin-based scaffold) compared to other cancer cells. MCF-7 cells adhere better on this scaffold, proliferate and form a spheroid structure. HeLa and HT-29 cells, on the other hand, may exhibit lower adhesion properties and therefore perform poorly in bovine skin gelatin-based scaffolds compared to MCF-7 [45]. MCF-7 cells are cells that can better penetrate porous structures and are prone to form spheroids with more cell-cell interactions. This porous structure contributed to better proliferation and infiltration of MCF-7 cells [1]. Breast cancer cells (especially MCF-7) are one of the cell lines that can mimic the 3D tumor microenvironment by forming spheroid structures. The homogeneous pore structure and optimal mechanical strength of bovin skin gelatin-based scaffolds supported the organization of these cells into 3D spheroid structures. HeLa and HT-29 cells, on the other hand, tend to grow in a monolayer planar manner and may not have formed strong spheroid structures like MCF-7. The better performance of MCF-7 cancer cell line on bovine skin gelatin-based scaffolds is associated with the high biocompatibility of bovin skin gelatin, its protein structure that enhances cell adhesion, its homogeneous porous structure and its microenvironment suitable for MCF-7's capacity to form spheroid structures. This created a tumor-like environment for MCF-7 cells, allowing the cells to show better proliferation, adhesion and 3D organization. HeLa and HT-29 cells were found to perform less well on these scaffolds due to their different cellular characteristics. Similarly, when we look at the literature, gelatin-based scaffolds give successful results especially with MCF-7 cell line. For example, Arya et al. reported that gelatin-based scaffolds provided a favorable environment for the growth of MCF-7 breast cancer cells and the cells showed good adhesion and proliferation [46]. Azar et al. found that fish gelatin showed lower thermal stability and mechanical strength, which may affect structural integrity in long-term cultures [47]. In our study, bovine gelatin offered a more stable and more suitable microenvironment for the cells in these aspects.

Cell culture is a widely used in vitro tool for the development of cell biology, tissue morphology and disease mechanisms, drug action, protein production and tissue engineering. Most cancer-related research is based on experiments using in vitro 2D cell cultures. However, 2D cultures have many limitations, such as changes in cell polarity, morphology and division method, and interaction imbalances between cellular and extracellular environments. These disadvantages led to the development of 3D culture models that can better mimic in vivo conditions [48]. Chen et al. found that MCF-7 cells were flat with triangular and polygonal morphologies after growing on 2D culture, while cells grown on collagen scaffolds had various morphologies including more rounded, spread out and elongated [49]. Findings obtained from studies conducted with cancer cell lines show that 3D systems are more advantageous in terms of proliferation and viability of cancer cells compared to 2D cultures [50–51]. The formation of cell aggregates or spheroids has been shown to better mimic tissue structure, although not all cells can come into direct contact with the medium due to limitations in nutrient and waste exchange [52]. The formation of these cell clusters and spheres in the studied 3D cultures may provide advantages to the models by providing different microenvironments for the cells [53].

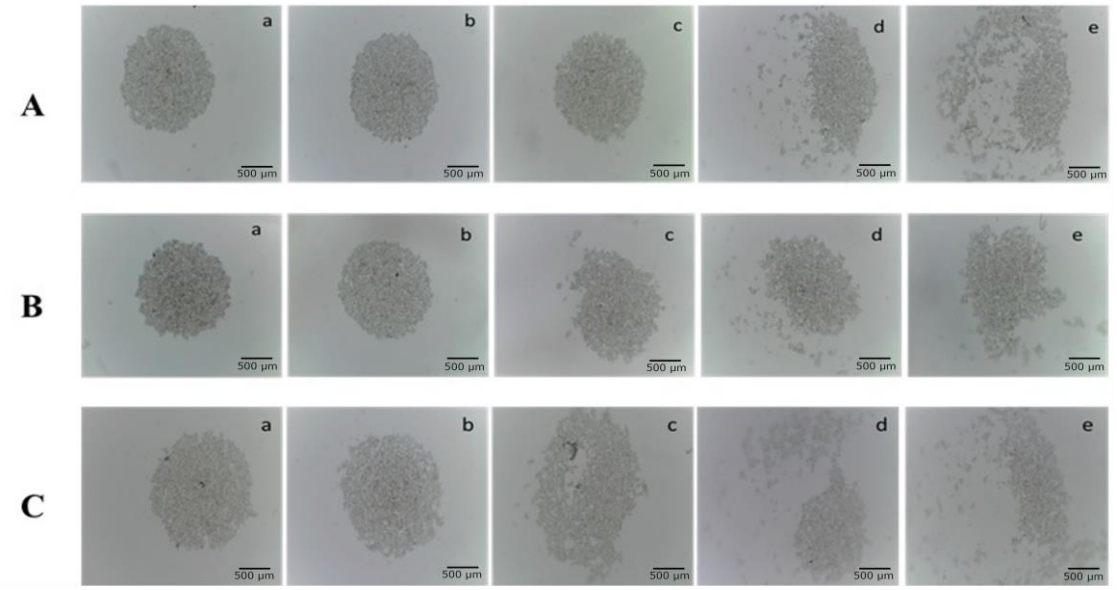

Fig. 5. Representative inverted microscopy images showing 3D spheroid formation of (A) MCF-7, (B) HT-29, and (C) HeLa cell lines cultured on various scaffold types after 7 days of incubation. All three cancer cell lines formed multicellular spheroid structures on the scaffolds. Morphological evaluation via inverted microscopy demonstrated that the cells predominantly exhibited a rounded, 3D architecture characteristic of spheroid morphology. (a) Agarose; (b) Phytagel; (c) Bovine skin gelatin; (d) Cold water fish gelatin; (e) Pig skin gelatin.

4. Conclusion

3D scaffold-based cell culture has become a crucial method in cancer research, offering a more accurate model to study tumor behavior, assess drug effectiveness, and explore interactions within the tumor microenvironment. Diverse scaffold materials such as hydrogels, polymers, decellularized matrices, and hybrid systems integrated with microfluidics have been engineered to create advanced and biomimetic 3D models. Notably, polymer-based scaffolds are distinguished by their adaptability, attributed to customizable mechanical properties and straightforward production methods.

This study focused on the development and characterization of innovative gelatin-based scaffolds for 3D cell culture. The analysis revealed a highly porous architecture with appropriate pore sizes in all compositions, promoting effective cell infiltration as well as nutrient and waste exchange. Additionally, in vitro evaluations confirmed that these scaffolds effectively support the formation of 3D cell spheroids.

Spheroid formation experiments were performed using HT-29, MCF-7, and HeLa cancer cell lines, all of which successfully grew on scaffolds, forming well-defined spheroid structures. Morphological analysis through inverted microscopy revealed predominantly 3D round cell morphologies, along with clear evidence of 3D cell-cell and cell-matrix interactions. Among the gelatin-based scaffolds, those prepared with bovine skin gelatin demonstrated the highest performance. The possible reason for this may be that bovine skin gelatin supports cell spreading due to its lower hardness. Furthermore, this study revealed that bovine skin gelatin-based scaffolds provide a more favorable microenvironment for optimal growth of MCF-7 cells in 3D culture compared to other cancer cell lines. This may be related to the stable pore structure, biocompatibility, mechanical strength and rich protein content of bovine skin gelatin, which promotes cell adhesion. Such scaffolds offer significant potential for biomedical applications such as 3D tumor modeling and drug testing. These results highlight that gelatin scaffolds offer an enhanced microenvironment for cancer cell proliferation. Collectively, the observed growth, proliferation, and spheroid

formation across all scaffold compositions demonstrate their promise for cell culture studies. Because these scaffolds can mimic the 3D structure of tumors, they can be used to more accurately study the natural behavior of cancer cells (proliferation, migration and invasion). They can be used in drug discovery processes by providing a more realistic environment to test the efficacy and side effects of anticancer drugs. Due to their biocompatible structures, they can be evaluated in drug delivery and controlled release systems and drug formulations due to their porosity. The developed scaffolds can promote cell infiltration, proliferation and ECM secretion to support regeneration of soft tissues (e.g. skin, muscle or connective tissue). These scaffolds enable the development of organoid models by preserving the phenotype of the cells and creating long-term biological structures. As a result, these scaffolds provide a promising platform for cell growth and tissue regeneration in biological and pharmaceutical research, preclinical testing, and tissue engineering applications.

Although this study successfully demonstrated the formation of 3D spheroid structures using various gelatin-based scaffolds, it has certain limitations that should be acknowledged. Firstly, the findings are based solely on in vitro assays, and in vivo validation has not yet been performed to confirm the physiological relevance and tumor-mimicking potential of the developed scaffolds within a living system. Secondly, while morphological observations and spheroid formation were assessed, no functional analyses such as apoptosis, cell viability (e.g., live/dead or metabolic activity assays like MTT), or gene/protein expression profiling were conducted to further elucidate the biological behavior of the cancer cells within the 3D microenvironment. Additionally, comparative evaluation was limited to traditional 2D monolayer culture in a general context, without detailed quantitative or functional benchmarking against 2D systems. In addition, using only MCF-7, HeLa, and HT-29 cell lines in this study does not sufficiently represent all cancer types or the biological diversity of tumors. Furthermore, since long-term culture results were not evaluated, it does not provide sufficient information about important parameters such as phenotypic stability, growth dynamics, and viability of the cells. These limitations indicate that the study's findings should be supported by more comprehensive tests in the future. Future research may explore the use of these scaffolds in therapeutic screening, in different types of cancer, in long-term 3D cultures, and broader research applications for validation.

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Ethical Approval

This study does not require study-specific approval by the appropriate ethics committee for research involving human subjects and/or animals.

Author Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Bahar Yılmaz and Mukaddes Keskinateş. Bahar Yılmaz: investigation, methodology, data curation, writing original draft, and validation. Esra Armağan: investigation, methodology, data curation, and writing original draft. Mukaddes Keskinateş: investigation, methodology, data curation, and writing original draft. Ziya Aydın: investigation, methodology, data curation, writing original draft, and validation. Mevlüt Bayrakcı: investigation, methodology, data curation, writing original draft, and validation. All authors read and approved the final manuscript.

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